

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE		3. REPORT TYPE AND DATES COVERED Final 03/15/92-03/14/95	
4. TITLE AND SUBTITLE Photoreceptors Regulating Circadian Behavior				5. FUNDING NUMBERS 61102F 2312-CS	
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Virginia P.O. Box 9003 Charlottesville, VA 22906				8. PERFORMING ORGANIZATION REPORT NUMBER AFOSR-TR-92-0419 1546001796A1	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Air Force Office of Scientific Research/NL Bolling AFB D.C. 20332-6448 Dr Haddad				10. SPONSORING/MONITORING AGENCY REPORT NUMBER F49620-92-J0205	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited.				12b. DISTRIBUTION CODE DTIC SELECTED JUN 16 1995 F	
13. ABSTRACT (Maximum 200 words) In mammals circadian responses to light are regulated by photoreceptors within the eye. Aged <i>rd/rd</i> mice (80 - 800 days) show unattenuated circadian responses to light but lack visual responses, while rodless transgenic mice (which lose rods earlier in development) show increased circadian responses to light. These data indicate that rod photoreceptors are not required for photoentrainment, but the early loss of rods may affect the development of the entrainment pathway. Aged <i>rds/rds</i> mice, lacking rod and cone outer segments, show circadian photosensitivities indistinguishable from <i>+/+</i> animals. The circadian system of aged <i>rd/rd</i> mice shows spectral responses that are consistent with the involvement of the known mouse cone opsins (green cone-511 nm; UV cone-359 nm). Low levels of both the green and UV cone opsin mRNA remain within the eyes of aged <i>rd/rd</i> mice. The subcutaneous eyes of the blind mole rat are used to regulate circadian responses to light. Preliminary data suggest that these eyes contain a single opsin that most closely resembles a green cone opsin. Collectively these data suggest that photoentrainment in mammals is mediated by cones. However, if the residual cones mediate circadian responses to light then very few are needed to maintain sensitivity and they do not require an outer segment.					
DTIC QUALITY INSPECTED 3					
14. SUBJECT TERMS				15. NUMBER OF PAGES	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT u		18. SECURITY CLASSIFICATION OF THIS PAGE u		19. SECURITY CLASSIFICATION OF ABSTRACT u	
				20. LIMITATION OF ABSTRACT u	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2/89)

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AFOSR Final Technical Report 1995

i) Original aims of the project.

In mammals, including man, light is the most effective agent that can be used to manipulate rhythmic physiology. However, the photoreceptors and their projections to central circadian oscillators remained poorly characterized. The long-term goals of my laboratory are to determine whether an identifiable chain of receptor cells, intra-retinal neurons, and their target retinal ganglion cells form an exclusive pathway conveying photic information for the synchronisation of the circadian clock. In this proposal we concentrated our efforts on determining which photoreceptor elements mediate circadian responses to light. As our experimental models we employed mammals that lack specific retinal elements and determined the effects of these defects on the regulation of circadian behaviour by light.

ii) Research Summary.

Circadian regulation by light in mammals is mediated by photoreceptors within the eye. Aged *rd/rd* mice (80 - 800 days) show unattenuated circadian responses to light but fail to respond to crude visual tasks. These data suggest that at some level circadian and visual responses to light are different. Rodless transgenic mice (which lose rods earlier in development than *rd/rd* mice) show increased circadian responses to light. Collectively these data indicate that rod photoreceptors are not required for photoentrainment, but the early loss of rods may affect the development of the entrainment pathway. Perhaps an altered input to the developing SCN permanently affects the response characteristics of the circadian oscillator to light. Aged *rds/rds* mice, lacking rod and cone outer segments, show circadian photosensitivities indistinguishable from *+/+* animals. These data demonstrate that photoreceptor outer segment are not required for photoentrainment. An action spectrum for phase shifting circadian rhythms of locomotor behavior in aged *rd/rd* mice shows spectral responses that are consistent with the involvement of the known mouse cone opsins (green cone λ_{\max} 511 nm; UV cone λ_{\max} 359 nm). In addition, low levels of both the green and UV cone opsin mRNA remain within the eyes of aged *rd/rd* mice. No rod opsin mRNA can be detected in these mice using PCR techniques.

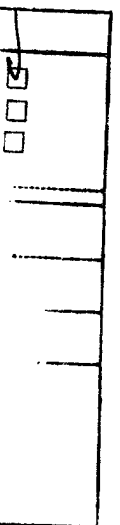
The blind mole rat uses it's minute subcutaneous eyes to regulate circadian responses to light. Our preliminary analysis of the eyes of this species suggest that these eyes contain a single opsin that most closely resembles a green cone opsin. These data, with our mouse results, suggest that circadian responses to light in mammals are mediated by cone opsins. However, if the residual cones mediate circadian responses to light, then very few are needed to maintain sensitivity and they do not require an outer segment. Alternatively, we cannot exclude the possibility that cone opsins in unidentified retinal cells mediate circadian responses to light.

Our findings in rodents showing that circadian behavioural responses to light remain unattenuated in animals which lack visual responses, and recent findings in humans, suggest that light entrainment mechanisms in mammals share fundamental similarities. For example, the photic regulation of pineal melatonin remains intact in certain groups of sightless people (Czeisler et al., 1995). As a result, the use of retinally degenerate mammalian models would seem to provide a powerful approach to the understanding of circadian organization in our own species.

iii) Research Results.

Daily or circadian rhythms are driven by endogenous pacemakers that have periods close to, but rarely exactly, 24 hours. In order to function adaptively, circadian systems must be synchronised (entrained) with the astronomical day by an environmental time signal. The majority of circadian rhythms are entrained by the irradiance changes at dawn and dusk. In mammals a circadian oscillator within the suprachiasmatic nuclei (SCN) and retinal photoreceptors provide the temporal information for the regulation of circadian physiology. Blinding or shielding the eyes from light blocks circadian responses to light in all mammals examined (Nelson and Zucker, 1981). Unlike all other vertebrates the mammalian pineal is not a photoreceptor. During the 1980s my laboratory demonstrated that the mammalian pineal contains a range of "photoreceptor proteins" (e.g. interphotoreceptor retinoid-binding protein, cellular retinal-binding protein, and opsin) but that it entirely lacks photopigment chromophore.

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We investigated whether 11-*cis* and all-*trans* retinaldehyde were present within the mammal pineal, and whether, if present, photoisomerisation of the 11-*cis* chromophore to the all-*trans* form could be demonstrated. Some 400 hamster pineals were collected and assayed for chromophore content, but none was found (Foster et al., 1989b) (c.f. avian or lizard pineals where chromophore can be extracted from relatively few pineals (Foster et al., 1989a; Provencio and Foster, 1993)).

Although we know that mammalian circadian photoreceptors are ocular, our detailed knowledge of the photoreceptors that regulate circadian physiology remained superficial. To a large extent our current understanding of the photoreceptors of the mammalian entrainment pathway stems from the work undertaken in this AFOSR supported grant. These results can be summarized as follows:

a) Circadian responses to light are unattenuated in aged retinally degenerate (*rd/rd*) mice that lack visual responses. The progression of photoreceptor degeneration in *rd/rd* mice commences early in postnatal development with the loss of rods, followed by a protracted loss of cones (Bowes et al., 1990). Circadian responses to light in animals 80 - 800 days of age were identical in *rd/rd* and *+/+* mice (Foster et al., 1991; Provencio et al., 1994). In addition, we have used the expression of the immediate early gene *c-fos* to address the possible impact of the *rd/rd* mutation on light activation of SCN cells. The distribution, number and density of Fos immunostained perikarya were identical in both *rd/rd* and *+/+* mice (Colwell and Foster, 1992). Circadian responses to light do not parallel either rod or cone cell loss in the *rd* retina. Note that blinding *rd* mice blocks all circadian responses to light. In conjunction with our analysis of circadian responses to light, we have also developed behavioural assays to assess the visual capabilities of retinally degenerate mice. The association of a bright light stimulus followed 5 seconds later by a mild electric shock was used as a behavioural assay of visual photoreception. Successful avoidance of the shock indicates an ability to detect the gross change in irradiance. In contrast to *+/+* mice, *rd* mice are incapable of learning to anticipate a shock which is immediately preceded by a bright pulse of light. The substitution of a 3kHz tone for the light pulse resulted in classical conditioning of both *+/+* and *rd* animals. These data emphasize the differences between "cognitive" and "circadian" light detecting tasks. In *rd* mice circadian responses to light are unattenuated, yet these animals fail to respond to even the crudest visual task (Provencio, et al., 1994).

b) Photoreceptor outer segments are not required for circadian responses to light. Another retinal mutation, *rds* (retinally degenerate slow) in the C3H mouse strain, has provided a second approach to the question of which elements in the eye mediate circadian responses to light. In *rds/rds* mice, the retina undergoes normal development and differentiation of cells until the first postnatal week, but at this time both rod and cone photoreceptors fail to develop outer segments and gradually degenerate. This is in contrast to *rd/rd* animals which develop outer segments that later degenerate. In *rds/rds* mice approximately half of all photoreceptor cell bodies have degenerated by 3 months and both rods and cones are equally affected. Virtually all photoreceptors are reported to be gone by 1 year of age. Our studies have shown that circadian responses to light are identical in *rds/rds*, *rd/rd* and *+/+* genotypes. This provides overwhelming evidence that the photoreceptive elements mediating circadian responses to light do not require an outer segment (Argamaso et al., 1995; Argamaso et al., 1993). These remarkable results raise the critical issue of how and where phototransduction can occur within the retina.

c) Rod photoreceptors are not required for circadian responses to light, but they do affect the photic entrainment pathway. Circadian responses to light were investigated in transgenic mice (Tm) with an integrated fusion gene consisting of a 1 kb fragment of the human rhodopsin promoter linked to the attenuated diphtheria toxin gene. Morphological and PCR analysis of retinæ has demonstrated that rods are eliminated between 2 - 28 days after birth, but cone cell bodies (with no outer segments) remain. Light pulses of varying irradiance (15 min., 515nm, circadian time 16) were administered to Tm and *+/+* mice and the magnitude of the circadian phase shift (min.) was correlated with light irradiance. Large differences were noted between the two groups, with Tm mice showing both an increased sensitivity (irradiance/response curve moved to the left) and abnormally large responses to light. At irradiances that produce saturating phase-shifts for *+/+* mice (103 min.), Tm mice showed phase shifts around 230 min.. In *rd/rd* mice, which lose rods later, we find only subtle increases in circadian photosensitivity. The different response kinetics of Tm, *rd/rd* and *+/+* mice could result from a reorganization of the entrainment pathway, and is more dramatic in the Tm than *rd/rd* mice because rods are affected earlier in postnatal development (Foster et al., 1995). The real basis for this

attenuated response has yet to be determined and will be addressed in future studies (see Appendix I).

d) The spectral sensitivity of circadian responses to light resemble cones: There is strong evidence for two types of cones within the mouse retina. Electroretinogram (ERG) and behavioural studies have shown two sensitivity maxima, a green-sensitive cone near 510 nm and an ultraviolet sensitive cone near 360 nm (Jacobs et al., 1991). We have completed an action spectrum for phase-shifting the circadian rhythm in aged *rd/rd* and *+/+* mice, and the data show two peak sensitivities, around 510 nm and 360 nm. On the basis of the similarity of our action spectrum results with the spectral sensitivity of the known cones, these photoreceptors become strong candidates for circadian regulation (Provencio and Foster, 1995) - paper appended.

e) The aged *rd/rd* mouse retina contains both the green and UV cone opsin mRNA. The genes and cDNAs for a rod opsin, UV cone opsin, and green cone opsin have been successfully characterized from the mouse retina. Using RT-PCR, followed by cloning and sequencing of the amplified cDNAs. We have succeeded in identifying low levels of both the green and UV cone opsin from the aged *rd* retina, but have failed to identify any rod opsin mRNA (animals > 2 years). These data, with our action spectrum results, implicate the cones in circadian regulation. It is worth stressing that although cone opsins and cell bodies remain in the *rd* retina, most of the cones have been lost and the remaining cones lack outer segments. If the residual cones do mediate circadian responses to light, then one must explain how responses remain unattenuated in aged *rd* mice. We recently proposed a model that could account for loss of photoreceptors with no loss in sensitivity (Foster, 1993).

f) An uncharacterised retinal mutation in mice results in attenuated circadian photosensitivities. A population of CBA mice have been identified which show retinal degeneration and have attenuated circadian responses to light when compared to wild-type CBA mice (Yoshimura et al., 1994). For example, mice exposed to a light pulse at an irradiance that causes 70% maximal phase shifts in normal mice (approximately 90 min.) will produce a phase shift of between 10 - 15 min. in retinal degenerate CBA animals. In addition, our preliminary results in these mice show that Fos induction in the SCN is robust in normal CBA animals but is little above background in these retinal degenerate mice. Further, Fos induction within retinal ganglion cells of these degenerate mice is much lower than that found in normal mice exposed to the same irradiances. The results suggest that the cause of attenuated photosensitivity would reside within the eye. The basis for this attenuated response has yet to be determined and will be addressed in future studies.

g) Cone opsins have been implicated in the regulation of circadian responses to light in the "blind" mole rat. The blind mole rat (*Spalax ehrenbergi*) is a highly specialized, subterranean mammal with rudimentary subcutaneous eyes. The retina of this species appears morphologically intact but contains reduced ganglion cell and photoreceptor populations. Previously we have identified a distinct molecular weight opsin in the retina (DeGrip et al., 1992), and most recently our preliminary molecular studies have isolated an opsin clone which shares considerable similarity to mammalian green cone opsins (Argamaso, et al., 1995). Furthermore, as a consequence of ocular regression in *Spalax*, all visual structures are severely hypoplastic, while the SCN and retinohypothalamic tract remain intact (Cooper et al., 1993a; Cooper et al., 1993b). Further, preliminary tract tracing studies in collaboration with Cooper (INSERM, Bron, France) have shown that all retinal ganglion cells project to the SCN. Preliminary data suggest that the photic regulation of circadian responses remains intact in *Spalax*, a mammal otherwise blind to light (Haim et al., 1983; Pevet et al., 1984). Collectively, these data support the hypothesis that *Spalax* has a "pure circadian retina", and could provide a very powerful model in the analysis of the light entrainment pathway.

iv) Future Work.

The results outlined above (iii a-g) have formed the basis of a series on future experiments that were initiated (but not completed) under the original grant. These approaches can be summarized as follows. Our strategy involves the continued use of mammals which possess a natural or genetic deletion of selected components of the visual system and assessing the effects of these "lesions" on circadian responses to light. This should allow the stepwise analysis of the discreet elements composing the entrainment pathway, from the retina to the circadian centers within the brain (SCN). Our future

studies will concentrate on three questions: 1) Do cone photoreceptors mediate circadian responses to light; 2) What are the intraretinal neurons and ganglion cells that compose the pathway from the retina to the circadian pacemaker within the SCN; 3) What role do rod photoreceptors play in the regulation of circadian physiology?

Question 1: Do cone photoreceptors mediate circadian responses to light? We will address this issue using three of our experimental models. Each model has different advantages, and collectively we should obtain an unambiguous answer to this question. In all three models we will be attempting to identify and localize opsins within the retina and implicate these opsins in the regulation of circadian physiology by using behavioural assays.

1a) Transgenic mice lacking green cone photoreceptors: Perhaps the simplest way to directly analyze the role of cone photoreceptors in circadian entrainment is to employ transgenic mice in which cones have been selectively eliminated. Our previous studies, in collaboration with Dr. Maureen McCall (University of Wisconsin), have examined transgenic mice in which the human rhodopsin promoter was attached to the attenuated diphtheria toxin gene (DTA), leading to the selective loss of rod photoreceptors (McCall et al., 1992). Dr. Jeremy Nathans (Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA) adopted a similar approach to eliminate the green cones in mice, and generously donated some of his founder animals to my laboratory. These animals were constructed in the following way: The Nathans group isolated the *cis* elements required for cone specific expression of transgenes. These promoter sequences were then fused to an attenuated DTA coding region (tox-176). Tox-176 encodes a toxin that contains a glycine to aspartate mutation in the NAD binding site. This mutation attenuates the activity of the toxin by approximately two orders of magnitude and allows for some "leakiness" in its expression. Fusion gene fragments containing the promoter and DTA coding region were injected into the pronuclei of C57BL/6 x C3H zygotes. The transgenic progeny of these injections were then identified by PCR analysis of DNA isolated from tail tissue. The PCR analysis used DTA specific primers. Those mice that carried the transgene (founder mice) were then used to produce founder lines. An initial screen for the presence of green cones involved crossing founder mice with transgenic mice in which the same promoter had been fused to the LacZ reporter. Offspring from this cross showed no expression of the reporter, but rod and UV cone opsins were identified using immunohistochemical techniques. These data suggest that green cones have been selectively eliminated in these animals. In our laboratory, two donated F2 males from the same founder line were bred to C57/BL normal females and to-date we have succeeded in producing 12 animals carrying the fusion gene construct. Dr. Nathans originally constructed these mice for subtractive hybridization studies to isolate cone cell specific mRNAs, and is not directly interested in the functional properties of these retinas. As a result he is eager for us to characterize the phenotype and analyze circadian photosensitivities.

Transgenic characterization: Using mouse specific primers, based on mouse opsin sequences, we have previously identified opsins within the retina of retinally degenerate mice and blind mole rats using RT-PCR (Reverse Transcriptase Polymerase Chain Reaction), including quantitative PCR approaches (Argamaso, et al., 1995). Eyes will be isolated and frozen in liquid nitrogen to prevent RNA degradation. RNA will be extracted using the AGPC method, and mRNA will be purified using oligo dT purification columns. cDNA will be synthesized from mRNA using reverse transcriptase. cDNA will be amplified with mouse opsin specific primers using quantitative PCR approaches. This analysis should confirm the absence of green cones in these mice and provide quantitative information of how the rods and UV cones have been affected by the transgene. In addition to our molecular characterization, we will study the morphology of the retina at the light and electron microscope level. The cytology of the remaining photoreceptors, and other retinal cells, will be examined to determine the effects of the transgene on retinal development and architecture. Other than the loss of green cones and perhaps some secondary loss of rods and UV cones, we do not expect any major changes in the retina. However anatomical verification is essential if behavioural results are to be put into any meaningful context.

Analysis of circadian responses to light: As in our previous experiments, assays of circadian photosensitivity will measure the magnitude of a phase shift in the freerunning locomotor rhythm produced by a single light pulse delivered when the mouse is of a defined age and level of retinal degeneration. Experimental animals are moved from the colony room into individual running wheel cages housed in light-tight boxes with time-clock controlled lights (Foster, et al., 1991). Currently we have facilities to house 96 mice under conditions that enable us to collect continuous

wheel running activity under a range of selected lighting conditions. At the heart of the system is a Dataquest III data acquisition system run on two IBM PS2s for continuous on-line activity recording. Data analysis occurs off-line using an IBM 486 microcomputer. The light cycle is terminated after 5-7 days and between the fourteenth and sixteenth day in darkness, each animal is removed from its cage using an infrared viewer, placed into the light pulse apparatus (described previously (Foster, et al., 1991)) and exposed to a 15 min. pulse of monochromatic light using an interference filter with a defined transmission and wavelength. The animal is returned to constant darkness for an additional 8 - 10 days. The pulses are delivered to each animal at specific times of the circadian cycle. The magnitude of the phase shift produced by the light pulse is measured as the difference between the steady state phase of the freerunning rhythm before and after the pulse (Foster, et al., 1991).

On the basis of our previous data we suggest that normally both the green and UV cones are capable of regulating circadian responses to light. The role of rods is ambiguous, they are certainly not required, but may have a role at some level (see Question 3 below). On the assumption that green cones have been entirely eliminated in our transgenic animals, and that normally rods play no role in circadian photoregulation we predict the following result in our transgenic animals: A 15 min. pulse of near-UV (360 nm) light will shift circadian rhythms of locomotor behaviour, but monochromatic light pulses outside the sensitivity range of UV-cones will not phase shift locomotor rhythms. If this is the case, then we will have strong evidence that cone photoreceptors opsins regulate circadian physiology. At this point *in situ* hybridization techniques (see below for methods) will be used to localize opsins within the transgenic retina. However, should we find that green light does cause phase shifts then we are left with several alternatives: 1) Not all the green cones have been eliminated in our transgenic animals. This should be determined from our molecular analysis (above). 2) Rod photoreceptors are capable of providing light information to the circadian system. We could test this possibility by breeding our green cone transgenics with either *rd/rd* mice or rodless transgenic mice, producing an animal lacking both rods and green cone. Circadian responses to light and molecular characterization would then be undertaken in these animals. 3) Another possibility would be that there is an unidentified photopigment (with green sensitivities) within the mouse eye that is capable of regulating circadian physiology. If this is the case then molecular techniques, RT-PCR using degenerate opsin primers or cloning a cDNA library made from rodless and green coneless eyes, will be used in an attempt to identify this "circadian" photopigment.

1b) The subterranean blind mole rat. The blind mole rat (*Spalax ehrenbergi*) is a highly specialized, subterranean mammal with rudimentary subcutaneous eyes (note that this animal is very different from the poikilothermic naked mole rat *Heterocephalus spp.*). The retina of the blind mole rat appears morphologically intact but with relatively few ganglion and photoreceptor cells. The visual areas of the brain have regressed, while the SCN and retinohypothalamic tract are well developed (Cooper, et al., 1993a; Cooper, et al., 1993b). Our recent tract tracing studies have shown that all retinal ganglion cells project to the SCN. Despite loss of visual function, circadian behaviour can be regulated by light (Haim, et al., 1983; Pevet, et al., 1984). Collectively, these data support the hypothesis that *Spalax* represents a species with a "pure circadian retina". Our preliminary molecular studies (RT-PCR) have isolated only a single opsin clone from this eye which shares considerable similarity to mammalian green cone opsins (Argamaso, et al., 1995). These preliminary data would support the hypothesis that circadian responses to light are mediated by cone-like photoreceptors. Our primary aim will be to isolate, localize and express the opsins from the mole rat eye, and implicate these opsins in the regulation of circadian physiology by determining the spectral sensitivity of circadian responses.

Blind mole rat eye cDNA expression library. The construction of a cDNA expression library will provide us with greater flexibility in the analysis of the photobiology of the blind mole rat eye. Upon successful isolation of the opsin(s), then other elements of the phototransduction cascade will be cloned from the library. In this way we hope to identify several of the key components of the phototransduction cascade of this "circadian" eye. RNA will be extracted from *Spalax* eyes by cesium trifluoroacetate gradient ultracentrifugation. mRNA will be purified on an oligo-dT-cellulose column. cDNA will be synthesized using the cDNA Synthesis System Plus (Amersham) and ligated into the pMOSElox vector (Amersham). After packaging with Gigapack II GOLD (Stratagene), the library will be plated immediately for the first screenings, in order to perform this with optimal recombinant representation. After plating of the entire library the plates will be overlaid with SM buffer, and the amplified library will be recovered and frozen in aliquots. Our cDNA library will be grown under conditions that induce the expression of fusion proteins, and will be screened with anti-opsin antibodies, including the antibodies that identified an opsin protein from *Spalax* eyes by Western blot

analysis. Partial cDNA clones that may be generated through expression screening and/or PCR cloning will be subcloned into plasmid vectors. These will be grown in *E.coli* cells, and the inserts will be purified, labeled with ^{32}P , and used as hybridization probes for screening of the cDNA library to obtain the full-length cDNA. Positive clones will be isolated, purified, subcloned and sequenced. Should this prove a successful approach for opsin analysis, then other members of the phototransduction cascade will be selected for cloning using the same strategy. We could then study the key elements of the phototransduction cascade of this remarkable eye. **Localization using *in situ* hybridization.** Any clones isolated will be used as probes for *in situ* hybridization studies. Currently we localize opsin mRNA in formalin-fixed tissue sections with digoxigenin-labeled RNA probes. These techniques have proved to be both reliable and remarkably sensitive. **Photopigment Expression.** We will utilize a baculovirus expression system, originally developed in the DeGrip laboratory (Nijmegen) for bovine rhodopsin (Jacobs, et al., 1991; Janssen, 1991; Janssen et al., 1988). This system can produce protein in sufficient quantity to study the biochemical properties of the pigments (spectral absorption, light-dependent changes, signal transduction). The biochemical properties of the isolated photopigments will be compared with the spectral sensitivity of circadian responses to light (below).

The spectral sensitivity of circadian responses to light in the blind mole rat: We have used both wheel running activity and measures of body temperature (implanted telemeters) as assays of circadian behaviour. Both measures work, although rhythms in body temperature have proved more robust and will be used in this study. We are in the process of constructing a phase response curve to light for the blind mole rat using a modified version of our light-pulse equipment (Foster, et al., 1991). As in our previous studies on the mouse, the primary assay of circadian photoreception will measure the magnitude of a phase shift in free running rhythms produced by a single light pulse delivered at a point in the phase response curve that produces maximum phase delays. Initially the absolute sensitivity of the photoreceptors mediating phase shifts will be characterized by measuring the effects of varying the irradiance of a monochromatic light pulse (515 nm) on the magnitude of the phase shift. We will then construct an equal photon action spectrum using 7 different wavelengths at a fluence that produced a half maximal response at 515 nm. This preliminary action spectrum will define the parameters for a relative quantum efficiency action spectrum that will be calculated from fluence-response relations at several different wavelengths. If there is a single photopigment, univariance will hold, and the fluence-response relations for the different wavelengths will be parallel. The eyes of *Spalax* are buried beneath the skin. As a result, the scatter and absorbance of light by overlaying tissue will need to be taken into consideration in the construction of the action spectrum. Similar approaches have been used in determining the spectral sensitivity of extraretinal photoreceptors in birds (Foster et al. 1985; Foster and Follett, 1985).

1c) CBA/J mice with attenuated circadian responses to light. In preliminary experiments CBA/J mice show attenuated circadian responses to light and attenuated levels of Fos induction within the SCN and retina. Our first aim is to confirm these results, then address the possibility that there may be a defect in the opsin mediating circadian responses to light. Our rationale for these experiments is based on the observation that the maximum sensitivity for phase shifting in CBA/J mice is near 470 nm. Our action spectrum in C57 *rd* mice suggests maximum sensitivities near 510 nm. This large discrepancy may be significant. We speculate that there may be a mutation of an opsin in CBA/J mice that alters both the spectral and absolute sensitivity of circadian responses to light. Our initial approach will isolate and sequence the green and UV cone opsins of the CBA/J mice and compare these sequences to those obtained in C57 *rd* and CBA/N mice. Any "mutated" opsins isolated will be expressed using the recombinant baculovirus-system and absorption spectra and photopigment dynamics determined. In addition, *in situ* hybridization techniques will be used to localize the defective opsin mRNA in an attempt to identify putative circadian photoreceptor cells. To isolate full-length cDNA clone(s) we will use RT-PCR and RACE (Rapid Amplification of cDNA Ends) analysis to determine the remaining 5' and 3' sequences of retinal opsin cDNAs. To determine the 3' sequence, cDNA will be synthesized using an oligo - dT primer with a 15 base pair tail. This tail subsequently becomes incorporated into the cDNA. PCR amplification is then performed using an opsin-specific and tail-specific primer. This PCR reaction will thereby amplify the 3' end of the deep brain opsin cDNA. To determine the 5' sequence, cDNA is synthesized using an opsin specific primer. This cDNA is purified and a 5' poly dC tail is added using Terminal Deoxy Transferase. PCR amplification is then performed using a second nested opsin-specific and tail-specific primer. This PCR reaction will amplify the 5' end of the opsin cDNA. The cDNA clones will undergo sequence analysis.

The power of this approach is that if we can link a defective opsin to abnormal circadian

responses to light, then we can implicate this opsin in the normal regulation of circadian behaviour. Further, if we localize this opsin we can then implicate a photoreceptor type in the regulation of circadian physiology. These constitute a simple set of experiments which could powerfully support those results obtained in coneless transgenic mice and the blind mole rat.

Question 2: What neurons and interneurons constitute the entrainment pathway? In parallel with the studies directed toward an identification of the photoreceptors mediating circadian responses to light, this part of the project will attempt to identify the complete light input pathway of the circadian system. Candidate photoreceptors will be identified using *in situ* hybridization techniques (discussed above) and retinal ganglion cells afferents and projections to the SCN will be identified by taking advantage of the infectious properties of the neurotropic porcine pseudorabies virus (Card et al., 1990; Card et al., 1991).

2a) Retino-hypothalamic tract characterization. Our primary approach will be to take advantage of the selective infection of the Bartha strain of porcine pseudorabies virus (donated by, Dr. J.P. Card, University of Pittsburgh, 446 Crawford Hall, Pittsburgh, PA 15260, USA). We have shown that injection of virus into the eye of the mouse or blind mole rat, leads to the selective labeling of hypothalamic neurons (including the SCN) and the intergeniculate leaflet of the lateral geniculate nucleus. The initial wave of infection leaves classical visual structures (DGN & tectum) uninfected. Subsequently the SCN will retrogradely label cells in the non-injected eye, offering the possibility of identifying the afferent retinohypothalamic ganglion cells. Our preliminary studies have identified labeled cells in the ganglion cell layer and in the inner nuclear layer of the retina of *rd* and *+/+* mice and blind mole rat. In the mouse we have identified a small sub-population of ganglion cells, while in the blind mole rat all ganglion cells are labeled by the virus. The central aim of this part of the project is to characterize these ganglion cells and determine the other retinal neurons that are in synaptic contact. This will involve the identification of retinal neurons as the infection spreads from the inner to outer layers of the retina. Classification of the different elements of the intraretinal circadian pathway will be based on morphological criteria (dendritic branching and stratification) and the use of antibodies directed to previously identified substances which can characterize ganglion cells, bipolar cells, and amacrine cells (MAP-1, protein kinase C, GABA, CHAT, tyrosine hydroxylase, somatostatin, substance P, etc...). These co-localization studies will employ double labeling immunohistochemistry with confocal microscopy of fluorescently labeled antibodies directed against the virus and the specific neuronal markers. Should we be successful in obtaining viral infection in the photoreceptor layer, then photoreceptors will be co-localised by employing either (1) conventional antibodies to rod and cone photoreceptors or (2) the opsin probes developed for *in situ* hybridization studies.

2b Examination of extra-SCN non-visual retinorecipient projections. Our preliminary studies in the mole rat and mouse suggest that, in addition to the SCN and intergeniculate leaflet, the "retino-hypothalamic" (RHT) pathway also innervates other brain structures. These centers include several lateral and hypothalamic areas, the bed nucleus of the stria terminalis (BNST) and the basal telencephalon. Using the viral tract tracing methods described above we aim to (1) determine whether retinal ganglion cells which innervate the SCN also innervate other non-visual areas (e.g. through axon collaterals) or whether a distinct class of ganglion cells connects these non-visual regions, and (2) identify the extra-SCN hypothalamic neurons receiving direct synaptic input from the retina. For the latter, the sensitivity of the viral tract tracing technique, combined with immunohistochemical demonstration of known peptidergic populations of neurons, should help to overcome the problem of identifying diffusely spread neurons within the hypothalamus. These RHT innervated cells, particularly in the basal telencephalon and BNST, are of considerable interest because they may constitute a wider network of neurons related to circadian organisation than previously considered.

Question 3: What role do rod photoreceptors play in the regulation of circadian physiology? Our rodless transgenic mice show both an increased sensitivity (irradiance/response curve moved to the left) and abnormally large responses to light (see Appendix I). These data show that rods are not required for circadian responses to light but their absence early in development is having a major impact on the organisation of the circadian system. At this stage we can only speculate at what level the circadian system is affected, and the aim of this part of the proposal is to define some of the effects of rod loss on the circadian axis. Our working hypothesis is as follows: If we assume that cones regulate circadian responses to light then the loss of rods early in retinal development will allow the cones to increase their input onto second order neurons (bipolar and horizontal cells). This results in an increase in the sensitivity of the circadian system to light. This increased retinal input to the developing

SCN permanently affects the response characteristics of the circadian oscillator (phase response curve), increasing the amplitude of phase delays and advances. Our experiments will attempt to find evidence for this hypothesis by asking two questions; a) In the absence of rods, do cones increase their input to the second order neurons? and b) Is there evidence of increased activation of retinal ganglion cells and SCN cells by light? These questions will be addressed as follows:

3a) In the absence of rods do cones increase their synaptic input to the second order neurons? Cone terminals have a distinct ultrastructure, consisting of a broad pedicle with several discrete synaptic elements, each with a separate synaptic ribbon. In several detailed studies (e.g. Sanyal, 1993), photoreceptor loss has been shown to result in an increase in photoreceptor inputs onto bipolar and horizontal cells. Adult (> 30 days) rodless transgenic mouse eyes and congenic, age matched, wild-type mouse eyes will be prepared for ultrastructural examination using standard techniques. Central and peripheral retina will be examined and the density of synaptic inputs onto bipolar and horizontal neurons will be determined (Jansen and Sanyal, 1992).

3b) Is there evidence of increased activation of retinal ganglion cells and SCN cells by light? The expression of the immediate early gene c-fos within the SCN and retinal ganglion cells has proved to be a useful marker of light activation of the circadian entrainment pathway. There is a robust correlation between the intensity of light, size of phase shift and levels of Fos expression within the SCN and ganglion cells of the retina (e.g. (Kornhauser et al., 1990)). This relationship will be examined in adult (> 30 days) rodless transgenic mice and congenic, age matched, wild-type mice. These methods have been described previously (Colwell and Foster, 1992). As loss of rods has increased the responses of the circadian system to light, we anticipate increased levels of Fos expression within both the retina and SCN. If there are no differences between transgenic and wild-type levels of Fos, then rod loss in the transgenics mice must be operating by some unidentified input pathway.

Full Publications resulting from grant (note that four full papers are in preparation):

Argamaso SM, Froehlich AC, McCall MA, Nevo E, Provencio I, Foster RG (1995) Photopigments and circadian systems of vertebrates. *BiophysChem* (in press):

Foster RG (1993) Photoreceptors and circadian systems. *Current Directions in Psychology Science* 2:34-39.

Foster RG, Argamaso S, Coleman S, Colwell CS, Lederman A, Provencio I (1993) Photoreceptors regulating circadian behavior: A mouse model. *Journal of Biological Rhythms* 8:S17-S23.

Foster RG, Argamaso S, DeGrip WJ, Garcia-Fernandez J, Provencio I (1993) Circadian photoreception in reptiles and mammals (Shima A, Ichihashi M, Fujiwara Y, Takebe H, ed), pp 267-272. Amsterdam: Elsevier.

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Provencio I, Foster RG (1993) Vitamin A₂-based photopigments within the pineal gland of a fully terrestrial vertebrate. *Neurosci Lett* 155:223-226.

Provencio I, Wong S, Lederman A, Argamaso SM, Foster RG (1994) Visual and circadian responses to light in aged retinally degenerate mice. *Vision Res* 34:1799-1806.

Provencio I, Foster RG (1995) Circadian rhythms in mice can be regulated by photoreceptors with cone-like characteristics. *Brain Reserarch* (in press):

Thesis either fully or partially supported from grant:

- 1) Ignacio Provencio. Ph.D. Will graduate, Summer 1995.
- 2) Sharleen Argamaso-Hernan. Ph.D. Will graduate, Summer 1995.
- 3) Bobby Soni. Ph.D. - in progress
- 4) Susan Doyle. Ph.D. - in progress

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Appendix I

RODLESS TRANSGENIC MICE SHOW INCREASED CIRCADIAN RESPONSES TO LIGHT.

Circadian responses to light were investigated in transgenic mice (Tm) with an integrated fusion gene consisting of a 1 kb fragment of the human rhodopsin promoter linked to the attenuated diphtheria toxin gene. Morphological and PCR analysis of retinæ has demonstrated that rods are eliminated between 2 - 28 days after birth, but cone cell bodies (with no outer segments) remain. Our original findings showed that freerunning circadian rhythms in constant darkness could be phase shifted by a single 70% saturating light pulse. At this irradiance, the magnitude of the phase shift in Tm mice was larger but within the normal range of +/+ mice of the same strain (Foster et al., Soc. for Neurosci. Abst., 1993). The relationship between irradiance and circadian responses in Tm mice has now been examined in greater detail. **Methods & Results** Light pulses (15 min., 515nm, 0.0001 - 10 $\mu\text{W}/\text{cm}^2$, circadian time 16) were administered to Tm and +/+ mice and the magnitude of phase shift (min.) was correlated with light irradiance (**Figure - Irradiance : Response Curve**). Large differences were noted between the two groups, with Tm mice showing both an increased sensitivity (irradiance/response curve moved to the left) and abnormally large responses to light. For example, at irradiances that produce saturating phase-shifts for +/+ mice (103 min.), Tm mice showed phase shifts around 230 min.. **Conclusions** In Tm mice, an absence of rods results in increased circadian responses to light, affecting both sensitivity and dynamic range. In *rd/rd* mice, which lose rods later, we find only subtle increases in circadian photosensitivity. The different response kinetics of Tm, *rd/rd* and +/+ mice could result from a reorganization of the entrainment pathway, and is more dramatic in the Tm mice because rods are affected earlier in postnatal development.

IRRADIANCE : RESPONSE CURVE

